Honors Thesis: The effects of temperature on the relative transcript amounts of carnitine palmitoyltransferase I in the liver and muscle of the japanese quail (*Coturnix japonica*) and the domestic pigeon (*Columbia livia domestica)*

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Abstract

The growth that takes place during the incubation period of all avians is a crucial time for the development of key internal systems and enzymes. When the regular temperature of a broiler egg is raised just 3° C higher than the optimal temperature for a two day period, there is slower embryonic development and a downward shift in the metabolism of lipids and carbohydrates (Willemsen et al, 2010). Willemsen's study, however, drew conclusions concerning metabolism based on the measurements of plasma metabolites and liver glycogen. This study aimed to determine the effects of intermittent high temperature on the relative transcript amounts of carnitine palmitoyltransferase I (CPTI), a key enzyme that facilitates the β-oxidation of fatty acids in the mitochondria. Carnitine palmitoyltransferase I transcript amounts were measured in breast muscle and liver tissue in the precocial Japanese quail (*Coturnix japonica*) and the altricial domestic pigeon (*Columba livia domestica*). Tissues were obtained from a previous study in which fertilized eggs were exposed to increased incubation temperatures (40.8°C) for three hours on days 10 and 11 of incubation for quail and days 13 and 14 for pigeon. The control eggs were kept at 37.6°C throughout incubation. Post hatch, the chicks were euthanized, and the liver and breast muscle were removed. The RNA was isolated from the tissue, reverse transcribed and the cDNA measured using real-time PCR.). Changes in the CPTI gene were compared to the housekeeper gene RPL-13. The results showed a numerical increase in quail liver, and a numerical decrease in relative transcript amounts of pigeon liver and muscle and quail muscle, but statistical differences were not detected.

Introduction

During the final stages of embryonic development, the liver is the most metabolically active organ as the chick prepares to hatch (Oliveira et al, 2008). Lipids are structural components of membranes and provide energy vital to energy homeostasis. (P. Nguyen et al, 2008). Long chain fatty acids (LCFAs) are integral to this homeostasis as they are broken down in the mitochondria through the β-oxidation pathway (Lopes-Marques et al., 2015).

The transfer of fatty acids to the inner mitochondria for oxidation occurs through multiple steps (Figure 1). LCFAs enter the cell and a Coenzyme A (CoA) group is added by the enzyme fatty acid acyl-CoA synthase (FACS), which forms a long chain acyl-CoA (Fillmore et al., 2010). When in this form, the long chain acyl-CoA is unable to permeate the membrane of the mitochondria. Carnitine palmitoyltransferase I (CPTI) is the enzyme that facilitates the attachment of the LCFA from CoA to carnitine, making long chain acylcarnitine (Lopes-Marques et al., 2015). This form of the acid is transported across the mitochondrial membrane via carnitine translocase (CAT); once it reaches the inner mitochondrial membrane, it is converted back into long chain acyl-CoA by carnitine palmitoyltransferase II (CPTII) and is oxidized. CPTI is regulated by malonyl-CoA, making CPTI the rate-limiting enzyme of fatty acid oxidation (Foster, 2012). CPTI comes in two different isoforms; CPTIa, the liver isoform found in most body tissues and CPTIb, the muscle isoform found mainly in heart and skeletal muscle and some adipose tissue (Schreurs et. al, 2010).

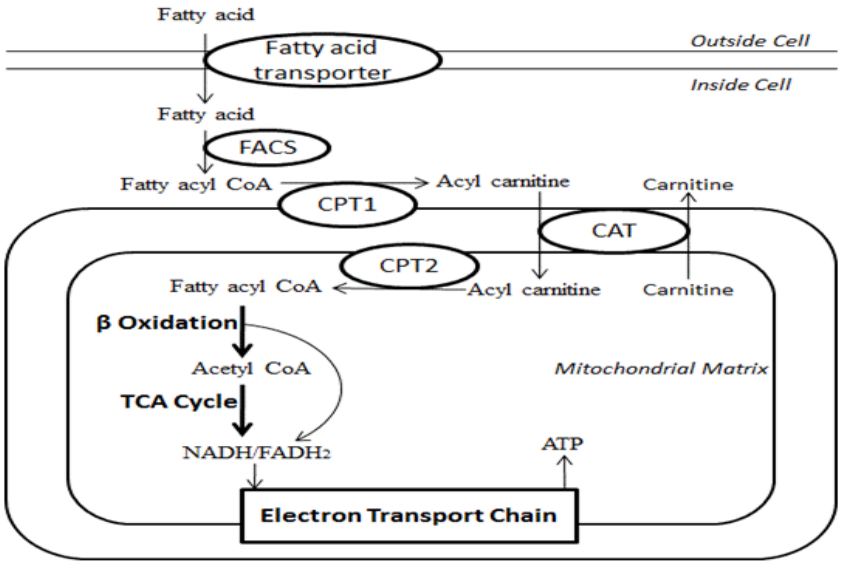

Figure 1.. Mechanism of fatty acid transport across the mitochondrial membrane (Fillmore et al., 2010).

The activity of CPTI is also dependent on the life stage of the animal and influenced by diet. During periods of fasting, the body initiates ketogenesis, a liver-specific process that increases fatty acid oxidation when glucose is limited (Foster, 2012). When fasting, the body senses a depletion of ATP due to a change in the ATP:AMP ratio which, under complex regulatory events, decreases the production of malonyl-CoA. This depletion of ATP and malonyl-CoA stimulates fatty acid oxidation, increasing the activity of CPTI (*unpublished,* Brockson et al.). Studies also show that CPTI activity is influenced by diet. When chickens were fed a high fat/low protein diet, the CPTIa in liver and CPTIb in muscle expression increased 1.5 to 2-fold and 2.5-fold, respectively compared to a low fat/high protein diet (Collin et al., 2009).

Since the development of birds takes place outside of the mother's body, their incubation environment significantly impacts development. Increased incubation temperatures can increase the hatching process, but also increase mortality (Willemsen et al., 2010). In Willemsen et al.'s study (2010), an increase in incubation temperatures by 3°C increased hatching time, reduced chick weight at hatch, and increased mortality. These findings were attributed to decreased carbohydrate and lipid metabolism as lipid absorption from the yolk remained unchanged (Willemsen et al., 2010) and agree with studies of lipid use in non-heat treated post-hatch chicks (Sklan, 2003). The objective of the current study was to evaluate high intermittent incubation temperatures on lipid metabolism of altricial and precocial birds. Specifically, this study aimed to determine if changes in CPT I may contribute to reduced lipid metabolism observed by others and as most studies have occurred in precocial birds, the current study also aimed to determine if embryonic heat exposure impacts altricial birds.

Materials and Methods

Tissue Collection and Storage. Quail and pigeon liver and breast muscle tissues were collected previously (*unpublished* Zappernick, 2016). Fertilized pigeon and quail eggs were weighed and allotted into a high heat or control temperature group. Eggs in the control group were incubated at 37.6°C (±0.01) with an average relative humidity of 56% (±0.31) for the entire incubation period. Eggs in the high heat groups were incubated initially at 37.6°C (±0.01) with an average relative humidity of 56% (±0.31), were transferred to a tabletop incubator set at 40.8°C (±0.10) with an average relative humidity of 56% (±0.49) at embryonic ages 10 and 11 days for the quail and 13 and 14 days for the pigeon. The eggs were incubated for 3 h, and then returned to the original incubator set at 37.6°C. All eggs were candled at day 8 and dead embryos were removed from the study. On day 16, all eggs were transferred to a tabletop incubator maintained at 37.8°C (±0.09) with an average relative humidity of 73% (±.004) for hatching. Chicks that emerged were euthanized and the breast muscle tissue without ribs and liver tissue were removed and stored in RNAlater® (Thermo Scientific Fisher).

RNA Isolation. RNA was isolated from liver and breast muscle using TRIzol® Reagent (Sigma). The concentration of mRNA per sample was determined by a NanoDrop™ Spectrophotometer (Thermo Scientific Fisher) and the quality of each sample was tested using agarose gel electrophoresis and ethidium bromide staining.

Reverse transcription and Primer Verification. Each RNA sample was reverse transcribed to cDNA using Omniscript® reverse transcriptase (Qiagen). Each reaction used a mixture of random hexamer and oligo dT primers and contained 1 ug of RNA within a final reaction volume of 20 uL.

Tissue specific primers for CPT Ia were designed using published NCBI sequence data for chickens (Accession number: NM_001012898.1) and the NCBI primer design tool. Primers for CPT Ib and RPL-13 were from a prior study. All primers were designed to span introns (Table 1). An optimal annealing temperature of 56.1°C was determined and confirmed through agarose gel electrophoresis.

Table 1: Primers used in the RT-PCR Reactions. All Primers except RPL -13 Liver were applicable with both species. All primers except for the CPTIa primer were the same in this study and the previous study.

Primer Name	5'-3' Forward Primer	5'-3' Reverse Primer
CPT-Ia	CTGTGGGATAGAAGACCTCTGA	GCCATTTTTGGAGAGAGTTCTGA
CPT-Ib	GACCTGGACAAGTACCCCGA	GCCCGCAATGATGTAGGAGA
RPL-13 Quail Liver	TGGCGGGCATTAACAAAAGG	TTGGCTTGCAGTGACTCTGT
RPL-13 Pigeon Liver	TTTCAAACGGGAGAAGGCCC	CCAAAGAGACGAGCGTTTGC
RPL-13 Muscle	TGGCGGGCATTAACAAAAGG	TTGGCTTGCAGTGACTCTGT

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Real-Time PCR. Real-time RT-PCR was performed using the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Inc). Fluorescence was detected in 96 well plates using Quantitect™ SYBR Green buffer (Qiagen, Inc.) for 45 cycles. Hot start TAQ DNA polymerase was

activated at 94°C for 15 minutes, followed by 45 cycles at 94°C for 15 seconds, 56.1°C for 30 seconds and 72.0°C for 30 seconds. Fluorescence data was collected at the end of the elongation cycle. Melting curve analysis was used to determine fluorescence as the temperature was raised from 50° C to 95°C in 0.5° C increments to detect non-specific amplification products. Samples were assessed in triplicate and no cDNA template controls were included in each run. The reactions were 25 uL containing 1 uL cDNA and 1.5uL each of the forward and reverse primers. All samples were run against the RPL-13 gene in liver and muscle, which was used as the internal control (Olias et. al, 2014).

Data analysis. Relative expression ratios signifying fold change of mRNA transcripts were determined according to Livak and Schmittgen (2001). For the quail, the variance of the expression ratios was calculated using standard error propagation methods. The mRNA relative transcript amounts of CPTI at high temperature were compared relative to the mRNA relative transcript amounts of CPTI of the control incubated birds. The results are shown as means ± SEM. Any differences were noted as significant when $P<0.05$.

Results

Transcript amounts of tissues exposed to high incubation temperatures relative to the control decreased 48% in pigeon muscle and decreased 14-fold in pigeon liver (Figure 1 and 2, respectively). In quail, transcript amounts of tissues exposed to high incubation temperatures relative to the control decreased 33-fold in quail muscle but increased 33% in quail liver (Figure 1 and 2, respectively). Using 95% confidence intervals, significant differences in relative transcript amounts for quail tissues were not detected. Analysis were not performed on pigeon tissues due to limited sample size.

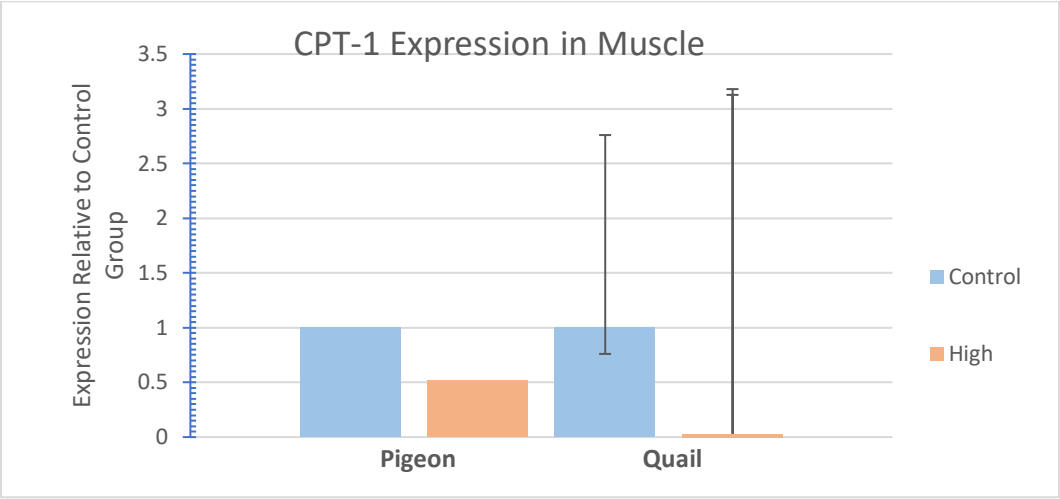

Figure 2: Relative CPTIb mRNA amounts in muscle of pigeon and quail. The control groups of each species were incubated at 37.6°C (±0.01) and the high temperature groups of each species were exposed to tabletop incubator set at 40.8°C (±0.10) at embryonic ages 10 and 11 for the quail and 13 and 14 for the pigeon and incubated for 3 h, and then returned to the original incubator set at 37.6°C (±0.01). The bars represent the relative fold gene expression with RPL-13 as the internal

control; control pigeon n=4, high pigeon n=2, control quail n=4 and high quail n=3. The error bars were calculated using a t-distribution critical value table based on the $2^{-\Delta\Delta CT}$ values calculated for each sample with a .05 significance level. Minor tick marks are shown at intervals of 0.05.

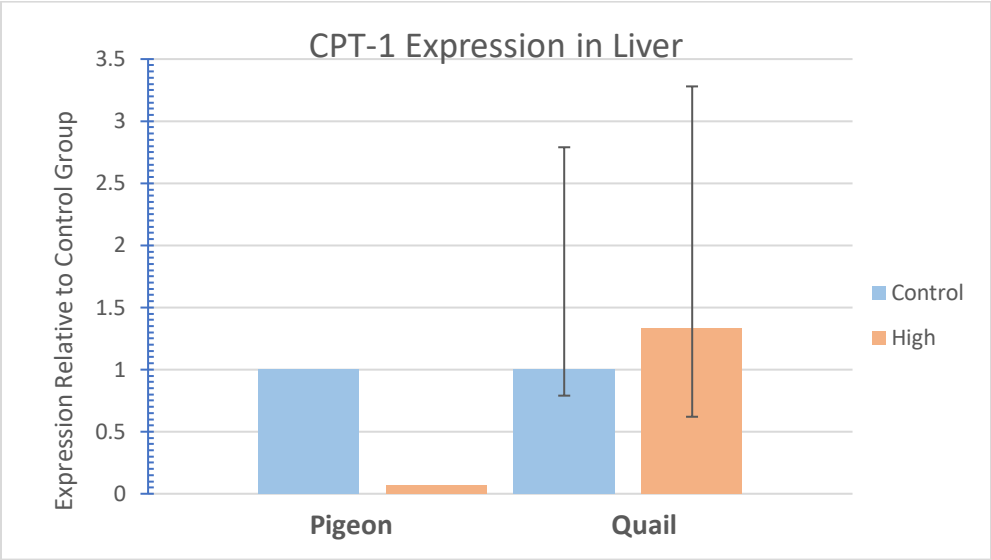

Figure 3: Relative CPTIa mRNA amounts in liver of pigeon and quail. The control groups of each species were incubated at 37.6°C (±0.01) and the high temperature groups of each species were exposed to tabletop incubator set at 40.8°C (±0.10) at embryonic ages 10 and 11 for the quail and 13 and 14 for the pigeon and incubated for 3 h, and then returned to the original incubator set at 37.6°C (±0.01). The bars represent the relative fold gene expression using RPL-13 as the internal control; control pigeon n=1, high pigeon n=1, control quail n=4 and high quail n=3. The error bars were calculated using a t-distribution critical value table based on the $2^{-\Delta\Delta CT}$ values calculated for each sample with a .05 significance level. Minor tick marks are shown at intervals of 0.05.

Discussion

From pre to post hatch, the metabolism of lipids and glucose changes drastically. Pre-hatch, fatty acid oxidation dominates as the main energy source to support rapid embryonic development (Sklan, 2003). Post hatch, the metabolism of lipids decreases significantly as circulating glucose increases and is used as the main energy source (Sklan, 2003). The decrease in lipid oxidation coincides with the hatching process as the oxygen in the egg becomes limited, thus limiting β-oxidation (Oliveira et al., 2008). As Willemsen et al. (2010) previously determined, high incubation temperatures can reduce glucose and lipid metabolism, which increases the chick's chance of death. Because CPTI is the rate limiting enzyme in lipid metabolism, it is an ideal target to study the mechanisms in lipid metabolism that are affected under heat stress conditions.

For the pigeon, the results in this study showed that the relative transcript amounts of CPTI were decreased compared to the transcript amounts of the control group in both liver and muscle. Further, previous studies from our lab noted that pigeons had a reduction in liver size (*unpublished,* Zappernick et. al) and a decrease in relative transcript amounts of

phosphofructokinase-1 (PFK-l), the rate limiting enzyme in glycolysis (*unpublished,* Loveless, 2017). Collectively, these observations coincide with Willemsen et al.'s (2010) data as well and may account for the reduced liver glycogen and reduced metabolism these authors observed.

During progressive starvation or fasting, glycogen levels in the blood become depleted and there is an immediate need for glucose especially for the brain. To conserve glucose for the brain, body-wide circulation of glucose is decreased and the body switches to fatty acid oxidation as the main source of energy. When food becomes more readily available and glucose circulation levels increase, fatty acid oxidation decreases. This flux between carbohydrate and lipid metabolism allows the body to have more than one source of energy and helps maintain caloric homeostasis (Gibson and Harris, 2002). A byproduct of carbohydrate and lipid metabolism is heat, and because the incubation temperature is already higher than the optimal temperature, the reduction in both metabolic pathways may be a response to reduce the internal temperature. But, this reduction in metabolism may be detrimental to embryonic development. The current study, and prior studies of our lab, suggest a decrease in both lipid and carbohydrate metabolism, suggesting that at high incubation temperatures overall energy metabolism is reduced during embryo development and may compromise birds at hatch. Indeed, in the previous study done by Zappernick et al. (*unpublished*) the pigeon hatchability was 47.6% and may be indicative of the embryo's inability to maintain homeostasis between carbohydrate and lipid metabolism.

The results for the quail in this study showed a decrease in relative transcript amounts for CPT I in muscle and an increase in the liver. These findings, in addition to the findings of a previous study measuring relative transcript amounts of PFK-l (*unpublished,* Loveless et al., 2017) suggest the liver and muscle of the precocial quail was adapting to achieve metabolic homeostasis and maintain energy for the hatched chick.

The current study shows that even a small increase in incubation temperature for a short period of time has the potential to change the relative transcript amounts of enzymes important to metabolism. Because of the small sample size for the altricial pigeon, it is tempting to speculate that temperature differences for even a few hours at a time may decrease lipid metabolism and hatchability, but further studies will be needed to confirm this. The results from the precocial quail, though not significant due to high error, suggest that an intermittent increase in temperature may not be as detrimental, but Willemsen et al.'s (2010) study shows that a high incubation temperature for a longer period may have a more significant effect on lipid and carbohydrate metabolism.

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